Exhibit G

Partial Hydrolysis of Poly(2-ethyl-2-oxazoline) and Potential Implications for Biomedical Applications?

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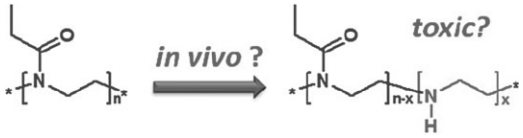
The hydrolysis of PEtOx is studied to evaluate the potential toxicity of partially hydrolyzed polymers that might interfere with its increasing popularity for biomedical applications. The hydrolysis of PEtOx is studied in the presence of digestive enzymes (gastric and intestinal) and at 5.8 M hydrochloric acid as a function of temperature (57, 73, 90, and 100 °C). It is found that PEtOx undergoes negligible hydrolysis at 37 °C and that thermal and solution properties are not altered when up to 10% of the polymer backbone is hydrolyzed. Mucosal irritation and cytotoxicity is also absent up to 10% hydrolysis levels. In conclusion, PEtOx will not decompose at physiological conditions, and partial hydrolysis will not limit its biomedical applications.



1. Introduction

Since many years, poly(ethylene glycol) (PEG), also called poly(ethylene oxide), is the gold standard to mask biomedical materials and devices from the immune system (“stealth” behavior) as well as to increase the half-life of pharmaceutical drugs, proteins as well as drug carriers in blood, which is known as PEGylation.[1–5] Despite this beneficial stealth behavior of PEG, there are also some drawbacks that limit more widespread usage, such as the preparation by living anionic polymerization complicating the incorporation of comonomers to tailor the properties or to incorporate side chain functionalities.[6] Moreover, PEG cannot be used as main excipient for implants or drug tablets due to its poor mechanical properties, i.e., PEG is a highly crystalline material with a melting temperature of 60 °C and a glass transition temperature (T_g) far below ambient temperature.

In recent years, poly(2-oxazoline)s (POX) received continuously increasing attention as potential biomaterials and as potential alternatives to PEG.[4,7–9] Similar to PEG, POX is non-toxic and is not recognized by the immune system of the body, which has been exploited to provide stealth behavior to drug carriers and surfaces of biomedical materials.[10–20] Moreover, the relatively easy access to well-defined POX via living cationic ring-opening polymerization of 2-oxazoline monomers provides direct access to copoly(2-oxazoline)s with tunable properties, such as solubility as well as solid state thermal properties. POX with methyl and ethyl side-chains are fully water-soluble at ambient and body temperature [although poly(2-ethyl-2-oxazoline) (PEtOx) has a lower critical solution temperature (LCST) of 60–65 °C; depending on molar mass],[21–23]

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polymers with propyl, *i*-propyl, or cyclopropyl side chains exhibit a LCST[24–27] below body temperature while larger aliphatic or aromatic side chains result in hydrophobic materials.[28] Moreover, the thermal properties of POX are widely tunable varying from amorphous materials with a T_g below ambient temperature ($T_g = -6\,^{\circ}C$) with branched aliphatic side chains,[29] via semicrystalline polymers with linear aliphatic side chains[30] to amorphous materials with a T_g of 140 °C with fluorinated aromatic side chains.[31] Importantly, both poly(2-methyl-2-oxazoline) and PEtOx are amorphous materials with a T_g of 80 and 60 °C, respectively, making them promising to be evaluated as pharmaceutical excipients.[32] Finally, a wide variety of side-chain and chain-end functionalized POX have been reported by the (co)polymerization of monomers carrying (protected) functional groups, such as alkenes,[33,34] alkynes,[35,36] carboxylic acids,[16,37] aldehydes,[38] and amines,[39,40] enabling straightforward bioconjugation.

One of the potential problems associated to the use of POX biomaterials might be the hydrolysis of the amide side chains resulting in linear polyethyleneimine (L-PEI), which is known to proceed under both strong acidic[41–44] and strong basic conditions.[45,46] Even though fully and partially hydrolyzed PEtOx are interesting materials for gene delivery and transfection, these (partially) hydrolyzed polymers, i.e., linear L-PEI or poly[(2-ethyl-2-oxazoline)-*co*-(ethylene imine)] (PEtOx-EI), do show cytotoxicity.[43,47,48] However, these reported cytotoxic PEtOx-EI copolymers comprised at least 56 mol% L-PEI. Finally, there has been one report suggesting partial hydrolysis of PEtOx in the presence of Proteinase K, although the amount of hydrolysis was not quantified.[49]

The aim of the current study is to evaluate the hydrolysis of PEtOx (Scheme 1) into detail in view of biomedical applications. In addition, the properties and cytotoxicity of PEtOx-EI with varying hydrolysis degrees is investigated to determine the degree of hydrolysis that is still tolerable for biomedical applications, which in combination with the hydrolysis rate will be very important for the future potential of POX as biomaterial. More specifically, the acidic hydrolysis of PEtOx is reported at various temperatures to assess the hydrolysis rate under ambient conditions. Moreover, a series of PEtOx-EI was prepared with controlled hydrolysis degrees. The thermal and solubility properties as well as the mucosal irritation and cell cytotoxicity of these copolymers will be discussed.

2. Experimental Section

2.1. Materials and Instrumentation

The commercially available PEtOx, Aquazol® 200 with $\overline{M}_w$ ~200 kDa, was obtained from Polymer Chemistry Innovations.

Deionized water was prepared with a resistivity less than 18.2 MΩ cm using an Arium 611 from Sartorius with the Sartopore 2150 (0.45 + 0.2 μm pore size) cartridge filter.

Simulated gastric fluid[50] was prepared by dissolving 2.0 g sodium chloride, 3.2 g porcine pepsine, and 7.0 mL of concentrated hydrochloric acid in deionized water followed by addition of deionized water to obtain 1000 mL solution leading to a final pH ~1.2.

Simulated intestinal fluid[50] was prepared by dissolving 6.8 g potassium dihydrogen phosphate in 200 mL deionized water followed by addition of 77 mL 0.2 M sodium hydroxide solution and another 500 mL of deionized water. Then, 10 g of pancreas powder, i.e., a mixture of amylase, lipase, and protease, was added, the pH was adjusted to pH = 6.8 and deionized water was added to obtain 1000 mL solution.

^{1}H NMR spectra were recorded on a 300 MHz Bruker Ultrashield. Chemical shifts are given in parts per million with respect to tetramethylsilane or residual solvent signals.

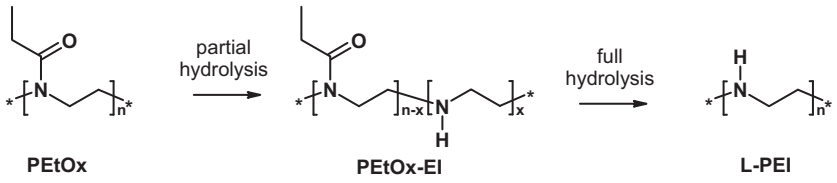
Size exclusion chromatography (SEC) was measured utilizing a Shimadzu LC-10AD pump, a waters 2414 refractive index detector (35 °C), a Spark Holland MIDAS injector, aPFG guard column followed by two PFG-linear-XL (7 μm, 8 × 300 mm^2) columns (PSS, Mainz, Germany) in series at 40 °C. Hexafluoroisopropyl alcohol (HFIP, Apollo Scientific Limited) with potassium trifluoroacetate (3 g · L^{-1}) was used as eluent (flow rate of 0.8 mL · min^{-1}). The molar masses were calculated against polystyrene standards.

The cloud points were recorded at 600 nm on a Varian Cary 300 Bio spectrophotometer equipped with Cary temperature control with two six-cell thermostatic cell holders with magnetic stirring device. The cloud points were measured with 5.0 mg · mL^{-1} polymer solutions (in deionized water, a basic NaOH solution with pH = 12.9 or an acidic HCl solution with pH = 1.0) and the investigated temperature range was 20–98 °C with heating and cooling ramps of 1 °C min^{-1} while stirring. All cuvettes were visually inspected after the heating program to facilitate the interpretation of the observed transmission profiles. The presented cloud point temperatures correspond to the precipitation temperature at 50% transmittance from the second heating curve. The polymer with 100% L-PEI was also measured with heating and cooling ramps of 0.1 and 0.017 °C min^{-1}.

Thermal transitions were determined on a differential scanning calorimeter (DSC) Perkin-Elmer 7 in hermetic aluminum cups containing 3–10 mg polymer. The samples were investigated in the temperature range from −40 to +100 °C with heating and cooling ramps of 20 °C min^{-1}, for both the glass transition temperatures and the melting points. One measurement is performed per sample after an initial heating run that was not considered for the subsequent calculations.

Thermogravimetric analysis (TGA) was performed on a TGA/SDTA851e, Mettler Toledo in a nitrogen atmosphere in a temperature range of 25–800 °C with a heating rate of 20 °C min^{-1}. Samples were dried under reduced pressure for 4 d at room temperature prior to the measurements.

Gas chromatography (GC) was performed on a GC8000 from CE instruments with a DB-5MS column (60 m × 0.249 mm × 0.25 μm) from J & W scientific. Injections were performed with a CTC A200S auto sampler and detection was done with a FID-detector. Injector and detector temperatures were kept constant at 250 °C.



Scheme 1. Schematic representation of the partial and full hydrolysis of poly(2-ethyl-2-oxazoline) (PEtOx) leading to poly(2-ethyl-2-oxazoline-*co*-ethylene imine) (PEtOx-EI) and linear poly(ethylene imine) (L-PEI), respectively.

2.2. Hydrolysis Kinetics of PEtOx

For the kinetic screening PEtOx (2.38 g) was dissolved in deionized water (25 mL) and after dissolution of the polymer, a 35 wt% hydrochloric acid solution in water (25 mL) was added resulting in an amide concentration of 0.48 and a 5.8 M HCl concentration. Directly after the addition of the HCl solution, the flask was placed in a preheated oil bath. Four different reaction temperatures were investigated, namely 100, 90, 73, and 57 °C (internal reaction temperatures). Samples were taken from the reactions at regular time intervals during 3–4 h and, subsequently, a concentrated NaOH solution was added to neutralize the pH to between 7 and 8 to ensure full deprotonation of the released propionic acid required to avoid evaporation. The solvent was removed by placing the sample vials on a hot plate at 100 °C and afterwards under reduced pressure. A small amount of deuterated methanol was added to the dried polymer and the hydrolysis degree from PEtOx to PEtOx-EI was determined by ^{1}H NMR spectroscopy using the signals from the released propionic acid ($-CH_2$ at $\delta = 2.1$) and the remaining CH_2 group in the side chain of PEtOx ($\delta = 2.4$).

2.3. Synthesis and Characterization of PEtOx With a Controlled Degree of Hydrolysis

Based on the above mentioned kinetic study, specific reaction temperatures and times were selected to reach 5, 10, 20, 50, 75, and 100% hydrolysis. These hydrolysis reactions were performed in a similar manner as described for the kinetic study, although no intermediate samples were taken. After the reaction, the mixture was cooled and the acidic solution was removed under reduced pressure. Then deionized water was added and the mixture was neutralized with 5% NaOH solution to $7 \leq pH \leq 9$ followed by purification by dialysis using a Spectra/Por® 6 dialysis membrane with molecular weight cut off of 500–1000 Da. After refreshing the solution surrounding the dialysis membrane several times, the final polymers were isolated by freeze-drying the content of the dialysis membrane.

The resulting partially hydrolyzed polymers were characterized by ^{1}H NMR spectroscopy in methanol-d_4, to determine the PEtOx-EI composition based on the signals from the secondary amine related signals ($-NH$ at $\delta = 4.0$ and CH_2N at $\delta = 2.7$) and the PEtOx side chain ($-CH_2$ at $\delta = 2.4$).

All polymers were analyzed by GC to ensure that no propionic acid was left, which might interfere with the biocompatibility studies. 12 mg of the polymers was weighed and dissolved in 200 μL methanol. A drop of trifluoroacetic acid (TFA) was added to protonate the propionic acid that might be present and this solution was injected into the GC. The temperature program started with an isotherm at 75 °C for 5 min, followed by heating to 200 °C with 10 °C min^{-1} and another isotherm at 200 °C for 1 min.

2.4. Slug Mucosal Irritation (SMI) Test

A slug-based assay to evaluate the mucosal irritation potential of the series of P(EtOx-*stat*-EI)s was used. This assay allows the assessment of mucosal tissue irritation in a simple yet efficient way without using large numbers of vertebrates such as mice, rabbits or non-human primates.[51] Laboratory slugs (*Arion lusitanicus*) were bred in plastic containers at 18–22 °C and fed with lettuce, carrots, cucumber, and protein rich food. Two days before the start of the experiment slugs having a body weight of 3–6 g were isolated. They were placed in a plastic box containing a paper towel moistened with phosphate-buffered saline (PBS) and kept at 18–22 °C. Only slugs without visible injuries were used during the experiment. The slugs were exposed, applied via gentle pipetting, for 1 h to the dissolved polymer (100 μL of a 10 mg mL^{-1} solution). Five slugs were used for each substance. The positive control was a 1% (w/v) solution of benzalkonium chloride (BAC) and the negative control was PBS (pH = 7.4).

The mucus produced during the contact period was measured by weighing the Petri dishes with the test component before and after the contact period and is expressed as percentage of the initial body weight. After 1 h the slugs were placed on a new Petri dish containing 1 mL PBS for 1 h. And again after 1 h the slugs were transferred to another dish containing 1 mL PBS. These two PBS samples were collected to assess lactate dehydrogenase (LDH) release.

The amount of enzyme that catalyzes the formation of 1 μmol · L^{-1} of NAD^+ per minute under the assay conditions is defined as one unit of LDH activity. The LDH activity (EC 1.1.1.27) was measured with an enzyme kit (LDH/HBDH 2.8, ABX diagnostics, Montpellier, France) and expressed as international unit per liter per gram body weight.

Mucus production was compared between the different treatments by one-way analysis of variance, after having confirmed that the mucus production was approximately normally distributed with the same variance in each of the treatment groups. Post-hoc analysis was based on Scheffe's procedure. A *p*-value below 0.05 was considered statistically significant. The analyses were conducted in SPSS version 17.

2.5. Cell Toxicity Tests

Cytotoxicity was assessed using an MTS assay (Promega), based on the bioreduction (dehydrogenase enzymes) of MTS [3-(4,5-

dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt], in the presence of phenazine methosulfate (PMS) as electron coupling agent, to formazan by metabolically active cells. The formazan production can be quantitatively monitored by measuring the absorbance at 490 nm and is proportional to the number of living cells. Human dermal fibroblasts (ATCC CRL-2522) were seeded in a 96 well plate at a density of 2.5×10^3 cells per well, allowed to attach overnight followed by incubation for 24 h with the respective polymer samples at a concentration of, respectively, 0.2, 1, and 5 mg · mL^{-1}. As positive control we used samples without cells and as negative control PBS was used.

An MTS/PMS solution was prepared by adding 100 μL of PMS solution (0.92 mg · mL^{-1} in PBS) to 2 mL MTS solution (2 mg · mL^{-1} in PBS with the pH adjusted to 6.0) followed by thorough vortexing. Subsequently, 20 μL of MTS/PMS solution was added to each well using a multichannel pipette and the plate was incubated for 3 h at 37 °C in a CO_2 incubator. Finally the absorbance at 490 nm was measured using a standard plate reader.

3. Results and Discussion

3.1. Hydrolysis Kinetics of PEtOx

The PEtOx hydrolysis was investigated for a commercially available PEtOx with a weight-average molar mass of 200 kg · mol^{-1} (PEtOx-200k) and a polydispersity index of 3–4, Aquazol® 200. ^{1}H NMR spectroscopic analysis only revealed the expected polymer signals and no impurities could be detected. This commercial Aquazol® material was chosen since PEtOx shows great promise for, amongst others, protective release formulations with tetrahydrocannabinol.[32] Even though this particular PEtOx-200k is not pharmaceutical grade, a pharmaceutical grade analogous material, namely Aquazol® High Purity, is also commercially available on request demonstrating the relevance of Aquazol® 200 as model polymer.[52] For internal use, the synthesis of pharmaceutical grade PEtOx still has to be developed under current good manufacturing practice (cGMP) conditions.

Initially, the hydrolysis of PEtOx-200k was investigated in simulated gastric fluid with pH = 1.2 containing 3.2 mg · mL^{-1} pepsine as well as simulated intestinal fluid containing 10 mg · mL^{-1} pancreatin; a mixture of amylase, lipase, and protease digestive enzymes. In both cases, the degree of hydrolysis was found to be negligible, i.e., less than 0.2%, after 6 h at 37 °C. Therefore, it can be concluded that hardly any hydrolysis will occur in the stomach for PEtOx based formulations and for in vivo applications of PEtOx in general; although additional long term in vivo studies will be required for usage of PEtOx in permanent implants.

To be able to estimate the hydrolysis rate at physiological temperature by extrapolation, accelerated PEtOx-200k hydrolysis was studied at 100 °C (internal reaction temperature) in 5.8 M HCl, revealing almost complete hydrolysis after 120 min (Figure 1, left), which is very similar to the hydrolysis kinetics of a shorter PEtOx demonstrating that the hydrolysis is not very much dependent on the chain length.[44] Since our aim is the evaluation of the hydrolysis at body temperature, the PEtOx-200k hydrolysis kinetics was also studied at three lower temperatures, namely 90, 73, and 57 °C (internal temperatures measured inside the reaction mixture), revealing that the hydrolysis rate strongly decreases with decreasing temperature (Figure 1, left). This strong temperature dependence is nicely illustrated by the hydrolysis conversion after 120 min, which decreases from 97% for 100 °C via 54% and 14% at 90 and 73 °C, respectively, to only 4% at 57 °C.

Previously, we demonstrated that the hydrolysis rate was independent on the polymer amide concentration ([A]) in solution indicating that the reaction follows pseudo-first order kinetics.[44] In addition, it may be assumed that the

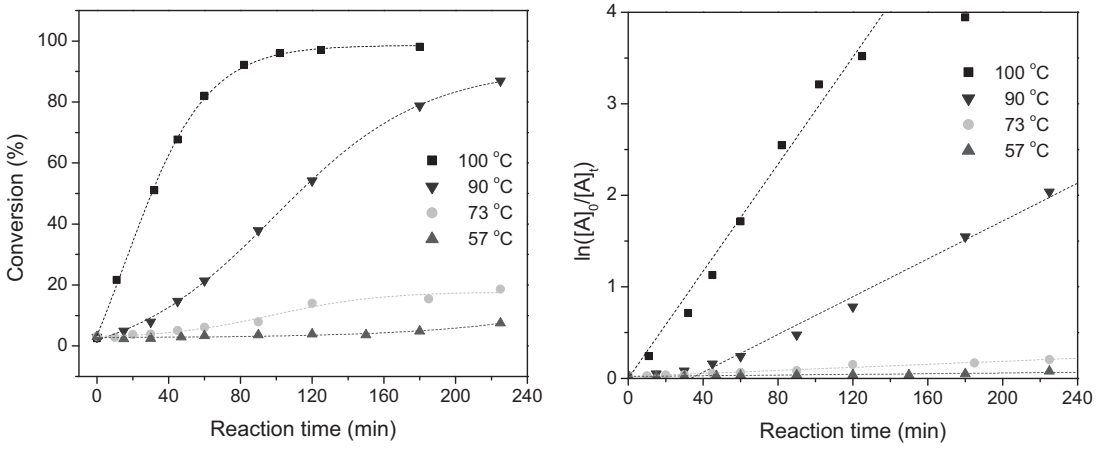


Figure 1. Left: Amide hydrolysis conversion in time for the acidic (5.8 M HCl in water) hydrolysis of PEtOx (sigmoidal fits are added to guide the eye). Right: First-order kinetic plot for the amide hydrolysis conversion together with linear fits.

Table 1. Hydrolysis rate constants (k_h) for the hydrolysis of PEtOx-200k.

Temperature [°C]	k_h [10^{-3} L mol^{-1} s^{-1}]
100	107 ± 2
90	23 ± 2
73	2.4 ± 0.2
57	0.6 ± 0.1

hydronium concentration ([H_3O^+]) is approximately constant during the reaction, because it acts as catalyst and is present in large excess ([H_3O^+] = 5.8 M and [A] = 0.48 M). The first order kinetic plot of ln([A]$_0$/[A]$_t$) versus time indeed shows an approximate linear relationship. The hydrolysis rate constants (k_h) are calculated from the slope of the linear pseudo-first order kinetic plot and the [H_3O^+] (Table 1). The activation energy for the PEtOx-200k hydrolysis was calculated to be 124 ± 13 kJ · mol^{-1} based on the Arrhenius equation. Therefore, the Arrhenius plot of ln k_h versus T^{-1} was prepared in which the slope corresponds to $-E_A/R$ (Figure 2). Extrapolation of the Arrhenius plot to 37 °C results in a calculated k_h of 2.6 × 10^{-8} L mol^{-1} s^{-1} allowing estimation of the required hydrolysis times to reach 1, 5, and 10% conversion at body temperature to be 40, 97, and 200 h, respectively. It is important to emphasize that it would thus take 40 h to reach 1% hydrolysis at pH = 0.75, which is both longer than the retention time in the stomach (maximum 4 h) and significantly more acidic than the stomach.

A more detailed look at the first order kinetic plots reveals an inhibition period that might be due to a neighboring group effect of already formed secondary amine moieties that catalyze the hydrolysis of nearby amide side chains; a similar effect was recently reported for the hydrolysis of (co)poly(2-oxazoline)s in ethanol-water solvent mixtures.[53] Based on this neighboring group effect, it may be expected that the hydrolysis does not occur in a truly random fashion but rather resembles the formation of polyethylene imine blocks throughout the polymer chains. The formation of such block distributions were also reported for the partial hydrolysis of poly(vinyl acetate) towards poly(vinyl alcohol).[54]

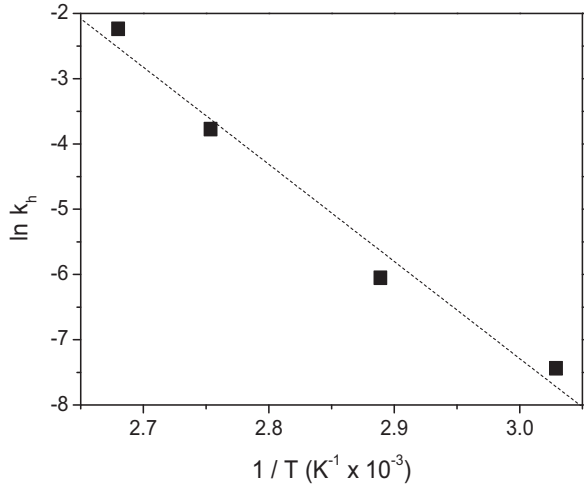


Figure 2. Arrhenius plot for the acidic hydrolysis of PEtOx-200k (5.8 M HCl).

3.2. Synthesis and Properties of Partially Hydrolyzed PEtOx

In addition to the hydrolysis kinetics under physiological conditions, it is important to understand the effect of partial PEtOx hydrolysis on the polymer properties to judge whether partial hydrolysis may interfere with biomedical applications. Therefore, a series of PEtOx-EI was prepared with a systematical variation in EI content varying from 0 to 100 mol%. The hydrolysis reaction conditions for obtaining the desired degrees of hydrolysis were selected based on the kinetic studies and are listed in Table 2 together with the characterization results.

To avoid the presence of a large quantity of salt that complicates purification, the hydrolysis solutions were evaporated to dryness directly after the reaction, so that most of the excess HCl was also evaporated. Subsequently, the polymer was redissolved in deionized water, followed by pH neutralization and purification by dialysis. The resulting isolated PEtOx-EI have compositions that are quite close to the target compositions, especially when taking into account that the copolymers were purified by dialysis that might have led to a change in composition due to size discrimination.

SEC analysis of the PEtOx-EI copolymers was performed using HFIP as eluent, which was found to be very well suited for the analysis of L-PEI (Table 2 and full SEC traces are shown in Figure S1).[44] The PEtOx, Aquazol® 200, revealed a broad bimodal molar mass distribution with a $\overline{M}_n$ of 35.5 kg · mol^{-1} and a PDI of 4.2. This bimodality of the molar mass distribution indicates that chain coupling occurred during the polymerization, most likely via chain transfer reactions leading to reactive enamine end-groups that couple at the final stages of the polymerization as previously reported.[55,56] Upon (partial) hydrolysis the calculated $\overline{M}_n$ increases in HFIP due to an increase of hydrodynamic volume in agreement with previous observations.[44] However, the $\overline{M}_n$ only increases up to 25% hydrolysis after which the $\overline{M}_n$ decreases again. Moreover, the PDI gradually decreases from 4.2 for PEtOx to 1.93 for L-PEI and the SEC trace is transformed into a monomodal molar mass distribution. Even though these observations indicate partial degradation of the polymer, our previous results clearly demonstrated that well-defined PEtOx with a

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Table 2. Hydrolysis conditions for the preparation of PEtOx-EI with varying EI content and structural characterization of the resulting copolymers.

Polymer	PEI content [wt%]	Theoretical degree of hydrolysis [%]	Hydrolysis temperature [°C]	Reaction time [min]	Experimental degree of hydrolysis [%]	$\overline{M}_{n,SEC}$ [g mol^{-1}]	PDI_{SEC}
PEtOx	0.0	0	–	–	0	35 500	4.20
PEtOx-EI-6	0.025	5	73	55	6	77 100	2.93
PEtOx-EI-9	0.041	10	73	111	9	95 500	3.06
PEtOx-EI-25	0.126	20	73	225	25	103 700	2.51
PEtOx-EI-43	0.247	50	100	32	43	81 700	2.58
PEtOx-EI-71	0.515	75	100	55	71	49 400	2.83
L-PEI	1.0	100	100	180	100	41 600	1.93

$\overline{M}_n$ of 15 kg · mol^{-1} and a PDI of 1.25 did not degrade, i.e., no change was observed in the shape of the molar mass distribution, upon full hydrolysis using similar conditions.[44] However, the presumed occurrence of chain transfer and chain coupling reactions during the synthesis of Aquazol® 200 lead to the coupling of polymer chains via amidic side chains, which can undergo hydrolysis resulting in a decrease in $\overline{M}_n$ and a change in the shape of the molar mass distribution as was also previously reported.[57]

The effect of partial hydrolysis of PEtOx on the thermal properties is important for the application of PEtOx in drug formulations, such as extrudates or tablet coatings. Therefore, the series of PEtOx-EI copolymers were analyzed by DSC and TGA (Table 3). TGA revealed that all (co)polymers degrade in one step and are stable up to at least 306 °C (5% weight loss), which is more than sufficient for storage of tablets and biomedical applications (see Figure S2 for the full TGA traces). However, TGA also revealed that partially hydrolyzed copolymers are more hygroscopic and contain some, i.e., 3 wt% for PEtOx-EI-6, water even after thorough drying of the solid polymer samples. The increased hygroscopicity of PEtOx upon partial hydrolysis can be ascribed to the strong hydrogen bond donating ability of L-PEI in combination with the strong hydrogen bond accepting capacity of PEtOx resulting in strong capturing of water. The presence of water might cause problems for both formulation as well as long term stability of water-sensitive drugs. The thermal transitions of the PEtOx-EI copolymers were determined by DSC (see Figure S3 for the DSC traces). PEtOx is an amorphous polymer with a glass transition temperature (T_g) of 61.8 °C while L-PEI is semi-crystalline with a T_g of −29.5 °C and a melting temperature (T_m) of 60 °C. All the partially hydrolyzed copolymers were found to be amorphous materials with intermediate T_g's (Table 3). However, when plotting the T_g's of the PEtOx-EI against the weight fraction of L-PEI present, it is evident that there is a deviation from the predicted T_g's using the Fox equation that assumes ideal mixing (Figure 3).

The PEtOx-EI with up to 25% hydrolysis unexpectedly have a T_g that is nearly equal to the PEtOx homopolymers

Table 3. Thermal properties of PEtOx-EI as determined by DSC (T_g and T_m) and TGA.

Polymer	L-PEI weight fraction	T_g [°C]	T_m peak [°C]	TGA 5% weight loss [°C]	H_2O[a)] [wt%]
PEtOx	0.0	61.8	–	402	0
PEtOx-EI-6	0.025	62.3	–	357	3
PEtOx-EI-9	0.041	n.d.	–	351	3
PEtOx-EI-25	0.126	58,9	–	332	4
PEtOx-EI-43	0.247	47.6	–	312	6
PEtOx-EI-71	0.515	5.1	–	306	16
L-PEI	1.0	−29.5	60	376	6

[a)]The amount of water is estimated by the decrease in weight around 100 °C.

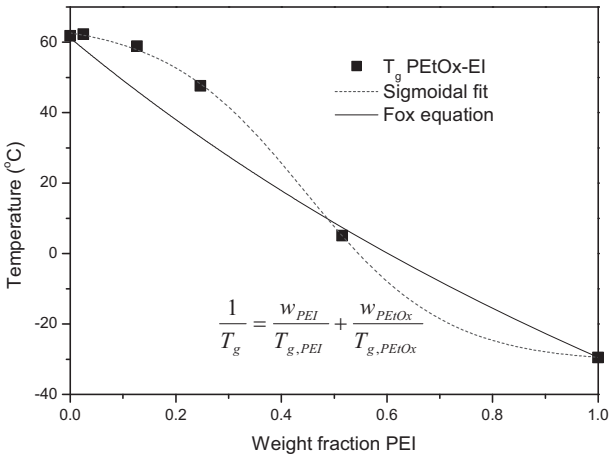

Figure 3. Glass transition temperature (T_g) of PEtOx-EI as function of weight fraction of L-PEI together with the expected T_g according to the Fox equation (see inset).

(PEtOx-EI-25 has a T_g of 58.9 °C), which demonstrates that partial hydrolysis will not be a problem for solid state applications of PEtOx as excipient for solid oral dosage forms. This strong positive deviation from the Fox equation with low PEI content clearly indicates attractive interactions between the PEtOx and L-PEI segments, which can be ascribed to hydrogen bonding between the secondary amine groups and amide groups. The PEtOx-EI-71 with 71 mol% L-PEI has a T_g that falls on top of the Fox equation indicating that the attractive forces are diminished with a too low amide content and that the more flexible ethylene imine units dominate the overall thermal behavior.

Since a large amount of current interest in POX for biomedical applications is directed towards the conjugation of POX to drugs, proteins or drug carriers to shield them from the immune system, the effect of partial hydrolysis on the solubility of PEtOx is of utmost importance. Since the solubility of PEtOx-EI can be strongly influenced by protonation of the amine groups, the solubility behavior was investigated by turbidimetry in acidic (pH ≈ 1), neutral (deionized water) and basic (pH ≈ 13) conditions (Table 4; Figure S4). These pH values were chosen to ensure full protonation or deprotonation of the copolymers, which is achieved at pH = 2 and 10, respectively.[58] The PEtOx homopolymers reveals a LCST transition, i.e., the polymer dissolves below the cloud point temperature (T_{CP}) and precipitates at the T_{CP}. The T_{CP} of PEtOx, Aquazol® 200, is 65 °C in deionized water and decreases to 57 °C in strong acidic or basic conditions due to the fact that the presence of ionic hydroxide or hydronium ions changes the ionic strength of the solution and, thus, the hydration behavior. Moreover, there is only a small difference in phase transition temperature between the heating and the cooling runs indicating the reversibility of the phase transition. In contrast to PEtOx, L-PEI is soluble in deionized water above 60 °C while below this temperature it forms insoluble crystalline hydrates.[59] Elevated temperatures are required to melt the hydrated crystals allowing dissolution of the polymer chains. This crystallization driven phase transition is accompanied by a relatively large hysteresis between heating and cooling, which strongly depends on the cooling rate (data not shown).[44] In basic solution, very similar behavior was observed for L-PEI while it is completely soluble in acidic solution due to full protonation that suppresses the crystallization.

In general, partial hydrolysis of PEtOx increases the solubility of the polymers in water. In fact, all PEtOx-EI copolymers are fully soluble in acidic water from 20 to 100 °C while in deionized water only the PEtOx-EI-6 revealed a T_{CP} at 84 °C. The increase in T_{CP} compared to PEtOx also indicates enhanced solubility, which can be ascribed to partial protonation of the secondary amine groups making the polymer more polar. In basic solution, the copolymers with up to 25 mol% hydrolysis revealed T_{CP}'s that increase with L-PEI content indicating that deprotonated L-PEI is slightly more hydrophilic than PEtOx.

The PEtOx-EI-43 showed a complex solubility behavior in basic solution with a bimodal decrease in transmittance, which indicates that stable aggregates are formed upon partial collapse of the polymer followed by full precipitation with further heating. Despite that the solubility behavior of the PEtOx-EI is dependent on the degree of

Table 4. Overview of cloud and clearance point temperatures of the PEtOx-EI copolymers measured at a polymer concentration of 5 mg · mL^{-1}.

Conditions		PEtOx	PEtOx-EI 6	PEtOx-EI 9	PEtOx-EI 25	PEtOx-EI 43	PEtOx-EI 71	L-PEI
acidic	heating	57	–	–	–	–	–	–
	cooling	55	–	–	–	–	–	–
neutral	heating	65	84	–	–	–	–	74
	cooling	64	79	–	–	–	–	51
basic	heating	57	60	61	69	–	–	76
	cooling	54	57	58	67	–	–	53

hydrolysis; all phase transition temperatures are higher than 50 °C and, thus, do not interfere with biomedical applications.

3.3. Mucosal Irritation and Cytotoxicity of Partially Hydrolyzed PEtOx

The effect of partial PEtOx hydrolysis on the biocompatibility was investigated based on in vivo SMI as well as in vitro cytotoxicity. The SMI assay is a relatively fast and simple method to assess mucosal tissue irritation as alternative to using higher species such as, e.g., rabbits. Furthermore, by quantifying mucus secretion by slugs upon contact with a test samples it was previously shown that sensitization in humans by the test samples can be predicted. In the current work, this slug based assay is related to in vitro cytotoxicity to evaluate its potential usage as predictive model for biocompatibility in addition to mucosal irritation. Therefore, an accelerated test protocol was adopted with relatively high polymer concentration and short exposure times.

For the SMI assay, the slugs were brought in contact with a solution of the PEtOx-EI (100 μL of a 10 mg · mL^{-1} solution). After 1 h exposure, the mucus production and mucus color were evaluated together with the release of LDH, which is indicative for mild tissue damage.[50,60,61] PBS and a 1% BAC were used as negative and positive controls. All synthesized (co)polymers were purified by dialysis before usage and the absence of propionic acid, that is released during the hydrolysis reaction, was confirmed by GC to avoid potential interference with the slug assay. For all the tested formulations, the mucus was colorless and there was no slug mortality demonstrating that no severe tissue damage occurred following a 1 h exposure with the solutions (Figure 4).

The PEtOx starting material was both tested before and after dialysis revealing that the as received PEtOx does contain some impurities, the mucus production of PEtOx before dialysis was on average 2% higher (95% CI, −0.2–4.3%) this is an indication of mild mucosal irritation. After dialysis, PEtOx and the PEtOx-PEI with up to 25% L-PEI content show minimal mucus production that was on average lower (although there was no evidence of a statistically significant difference, $p > 0.05$) in comparison with the negative control indicating that they are non-irritating. Moreover, these formulations did not induce LDH release indicating no tissue damage. The polymers with higher L-PEI content resulted in a mucus production that was statistically comparable with the negative controls ($p > 0.05$). Since PEtOX-EI-43 and PEtOX-EI-71 induced LDH release in one out of five slugs this might be an indication that longer exposure will probably result in tissue damage. Finally, it was found that commercially available hyperbranched PEI (HB-PEI) shows a slightly higher mucus

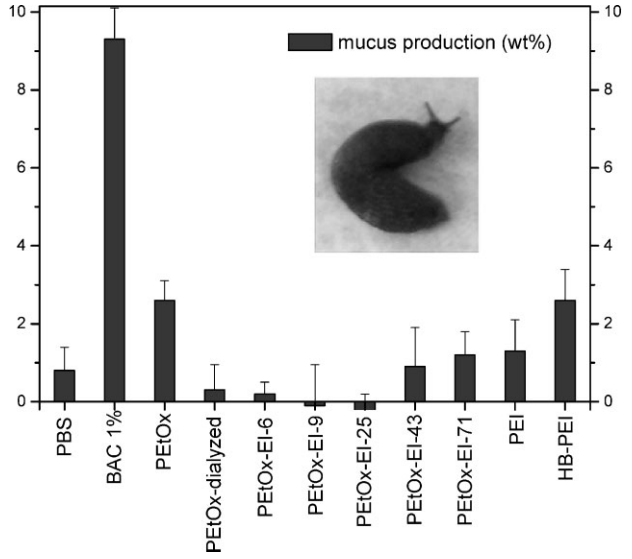

Figure 4. Mucus production and LDH release obtained during the slug (see picture) mucosal irritation assay on PEtOx before and after dialysis, the PEtOx-EI copolymers, hyperbranched PEI (HB-PEI), PBS as negative control, and a 1% benzalkonium chloride solution (BAC 1%) as positive control.

production than L-PEI, which can be ascribed to the presence of more basic tertiary amine groups as well as the better solubility of HB-PEI since L-PEI had to be added as a milky suspension due to incomplete solubility. In summary, this SMI assay revealed that up to 25% of PEtOx hydrolysis may occur before the material becomes mildly irritating.

The cytotoxicity of the PEtOx-EI was evaluated by exposure to human dermal fibroblast cells for 24 h (Figure 5). The PEtOx with up to 25% partial hydrolysis show no substantial cytotoxicity up to 1 mg · mL^{-1} concentration and a still relatively high cell viability of

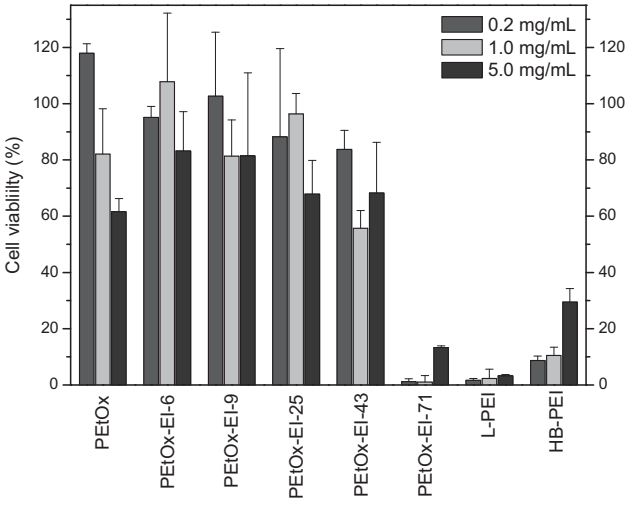

Figure 5. Cell viability of human dermal fibroblast cells upon 24 h exposure to solutions of PEtOx, PEtOx-EI, L-PEI, and HB-PEI.

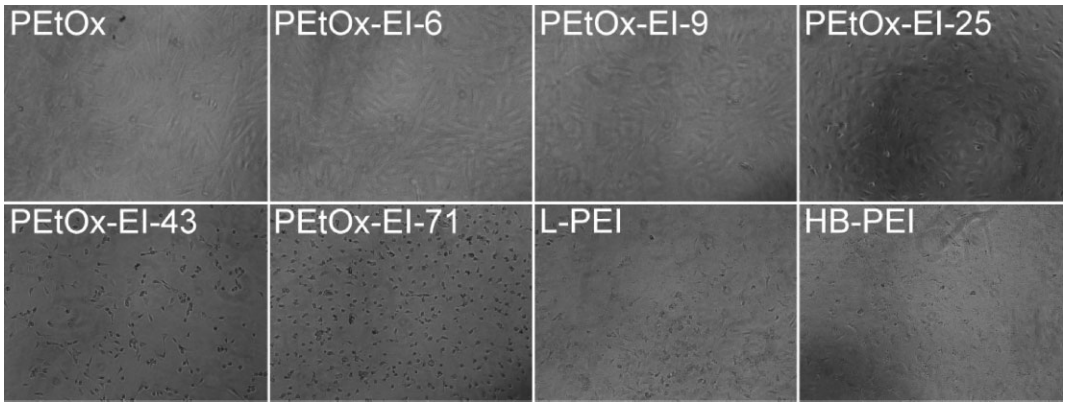

Figure 6. Phase contrast images (700 × 500 μm²) of human dermal fibroblast cells after 24 h exposure to 5 mg mL^{-1} solutions of the (co)polymers.

around 80% for 5 mg · mL^{-1} concentrations, which is very high for drug delivery applications. PEtOx-EI-43 shows slightly lower cell viability while higher degrees of hydrolysis result in rather low cell survival rates indicating severe cytotoxicity. In contrast to the SMI, the L-PEI is more toxic to the human dermal fibroblast cells than HB-PEI. In addition to the cell viability, the appearance of the cells was evaluated by phase contrast microscopy after 24 h exposure to 5 mg · mL^{-1} polymer (Figure 6). The cells are nicely stretched and hardly visible for PEtOx, PEtOx-EI-6 and PEtOx-EI-9 indicating that the presence of the polymers does not influence the cells. For PEtOx-EI-25 some cells rolled up indicative of a cytotoxic effect of the polymer. With 43% L-PEI or more all cells all rolled up indicating that the polymers are toxic to the cells. For L-PEI and HB-PEI, only debris of dead cells could be observed. From these cell studies it can be concluded that PEtOx with up to 9 mol% hydrolysis is not toxic to the cells even up to a concentration of 5 mg · mL^{-1}. Since the SMI assay revealed that PEtOx with up to 25 mol% hydrolysis were non-irritating, it can be concluded that the slug assay may be used as preliminary predictive model for cell toxicity.

4. Conclusion

The partial hydrolysis of PEtOx is demonstrated to hardly occur under biologically relevant conditions, i.e., acidic conditions and/or the presence of enzymes at 37 °C, which is an important prerequisite for further development of biomedical applications based on PEtOx. Moreover, a series of PEtOx-EI with a controlled degree of hydrolysis was prepared and characterized with regard to thermal and solution properties. DSC revealed that up to 25% hydrolysis may occur before the T_g is significantly lowered while TGA demonstrated that the thermal stability is very good with a 5% weight loss temperature higher than 300 °C regardless of the degree of hydrolysis. Even though PEtOx and some PEtOx-EI copolymers do reveal LCST behavior, this will not interfere with biomedical applications since the T_{CP} is higher than 50 °C. Only very high degree of hydrolysis might be a problem for biomedical applications, because this will lead to insolubility at low temperatures due to crystallization. More importantly, a SMI assay revealed that polymers with less than 25% hydrolysis do not show any mucosal irritation or tissue damage. Additionally, human dermal fibroblasts were unaffected by 24 h exposure to 5 mg · mL^{-1} solutions of PEtOx with less than 10% hydrolysis for 2 h. These latter results also indicate that the SMI assay does provide a preliminary prediction for cytotoxicity; despite being less sensitive.

All together, it is demonstrated that PEtOx can be safely used for in vitro and in vivo biomedical applications, since partial hydrolysis will hardly occur. Moreover, even when partial hydrolysis would occur it does not pose problems with regard to changes in material properties nor cytotoxicity if it remains below a degree of hydrolysis of 10%. These combined results, thus, demonstrate that in vivo PEtOx degradation will, most likely, not be a limiting factor for biomedical applications.

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